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(54) Title: METHOD OF PROMOTING NEURONAL CELL PROLIFERATION AND DIFFRENTIATION

(57) Abstract

The present invention provides methods, improved cell culture medium and kits for promoting neuronal cell proliferation and/or differentiation by growth in the presence of angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and/or AII AT_2 type 2 receptor agonists, either alone or in combination with other growth factors and cytokines.

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METHOD OF PROMOTING NEURONAL CELL PROLIFERATION AND DIFFERENTIATION

Cross Reference

This application is a continuation in part of U.S. Application Serial No. 60/075,232 filed February 19, 1998.

Field of the Invention

This present invention relates to methods and kits for accelerating the proliferation and/or differentiation of neuronal cells.

Background of the Invention

Stem cells in the central nervous system (hereinafter referred to as "CNS") have the potential to differentiate into neurons, astrocytes, and oligodendrocytes and to self renew. (McKay, *Science* 276:66-71, 1997; hereby incorporated by reference in its entirety). CNS progenitor cells have a more restricted potential than a stem cell, while CNS precursor cells comprise any non-fully differentiated CNS cell type (McKay, 1997).

Mammalian fetal precursor cells that give rise to neurons and glia have been isolated (Frederiksen et al., *Neuron* 1:439 (1988); Reynolds and Weiss, *Science* 255:1707 (1992); Davis and Temple, *Nature* 372:263 (1994)). The adult CNS also contains multipotential precursor cells for neurons, astrocytes and oligodendrocytes (McKay, 1997). Cultured cells from both the fetal and adult CNS that have proliferated *in vitro* can differentiate to show morphological and electrophysiological features characteristic of neurons (Gritti et al., *J.*

Neurosci. 16:1091 (1996); Vicario-Abejon et al., *Neuron* 15:105 (1995)). These data show the multipotential nature of cells derived from the CNS.

Multipotential cells from the fetal brain have been demonstrated to be both homogenous and stable. (Johe et al., *Genes Dev.* 10:3129 (1996)). *In vitro*, these cells divide daily and efficiently generate neurons and glia for at least the first month of culture. These cells can be considered to be stem cells because they fulfill the criteria of multipotency and self-renewal.

Cells from the adult brain proliferate and differentiate into neurons and glia in tissue culture with the same efficiency for neuronal differentiation as found in fetal stem cells and the same response to extracellular ligands (McKay, 1997). Thus, similar general mechanisms control the differentiation of stem cells from fetal or adult brain. The proliferation of precursor cells in the adult forebrain can be stimulated by the direct application of mitogenic growth factors *in vivo*, and in animals treated in this way, proliferating cells in the subventricular zone differentiate into neurons and glia (Craig et al., *J. Neurosci.* 16:2649 (1996)). However, *in vivo* less than 3% of the proliferating cells labeled with bromodeoxyuridine differentiate into neurons (McKay, 1997). The discrepancy between the efficient neuronal differentiation of adult stem cells *in vitro* and their inefficient differentiation *in vivo* is a critical but unresolved question in the field (*Id.*) The lack of differentiating neurons may not be a consequence of the lack of cells with the appropriate potential but rather a function of the signaling environment in the adult brain (*Id.*)

The long term delivery of proteins in the brain is a major goal in gene therapy. Transplantation of cells engineered to produce growth factors shows the potential of grafted cells as vectors for protein delivery (Beck et al., *Nature* 373:339 (1995); Tomac et al., *Nature* 373:335 (1995); Moore et al., *Nature* 382:76 (1996)). It is possible to generate many different

immortal cell lines from the developing CNS. It is also possible to graft primary cells expanded *in vitro*. Experiments have suggested that primary adult cells derived from the hippocampus and cultured for long periods *in vitro* can still differentiate into neurons when re-implanted into the migratory pathway used to replenish neurons in the adult olfactory bulb (Suhonen et al., *Nature* 383:624 (1996)). Experimental grafts in animal models suggest that the integration of grafted neurons into the circuitry of the host may be possible, as other studies illustrate the use of *in vitro* manipulated donor cells that differentiate *in vivo* into oligodendrocytes (Tontsch et al., *Proc. Natl. Acad. Sci.* 91:11616 (1994); Groves et al., *Nature* 362:453 (1993)). Furthermore, clinical trials show that neuron replacement therapies for neurodegenerative diseases, such as Parkinson's and Huntington's disease, are feasible (Kordower et al., *New Engl. J. Med.* 332:1118 (1995); Lindvall et al., *Ann. Neurol.* 35:172 (1994)). Thus, for clinical applications, cell culture offers an important opportunity to use sophisticated genetics in cell-based therapies for neural disease (McKay, 1997).

CNS stem cells have been expanded in vitro by using epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (McKay, 1997). *In vitro*, EGF has also been shown to be a differentiation factor for astrocytes.

While these studies demonstrate the potential for stem cell and neuron replacement therapy, a careful analysis of adult neuronal stem cells has only just begun. Further characterization of the mechanisms that control the multipotentiality, self-renewal and fate restriction of neuronal stem cells is clearly important to develop new therapies for cell regeneration and replacement in the adult nervous system. (Johe et al., 1996.)

Methods that increase the *in vitro* and *ex vivo* proliferation and differentiation of neuronal stem and progenitor cells will greatly increase the utility of neuron replacement therapy in various neurodegenerative conditions such as Parkinson's and Alzheimer's

Diseases, and amyotrophic lateral sclerosis. Similarly, methods that increase *in vivo* proliferation and differentiation of neuronal stem and progenitor cells will enhance the utility of neuron replacement therapy by rapidly increasing local concentrations of neuronal stem and progenitor cell at the site of therapy.

Summary of the Invention

The present invention provides methods that increase the proliferation or differentiation of neuronal stem and progenitor cells that are useful in rapidly providing a large population of such cells for use in neuron replacement therapy and for making a large population of transfected cells for use in neuron replacement therapy.

In one aspect, the present invention provides methods that promote neuronal cell proliferation or differentiation by contacting the cells with angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II (AII), AII analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists, either alone or in combination with other growth factors and cytokines.

In another aspect of the present invention, an improved cell culture medium is provided for the proliferation or differentiation of neuronal cells, wherein the improvement comprises addition to the cell culture medium of an effective amount of angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists.

In a further aspect, the present invention provides kits for the propagation or differentiation of neuronal cells, wherein the kits comprise an effective amount of angiotensinogen, AI, AI analogues, and/or AI fragments and analogues thereof, AII, AII analogues, AII fragments or analogues thereof, and/or AII AT_2 type 2 receptor agonists, and

instructions for culturing the cells. Preferred embodiments of the kit further comprise cell culture growth medium, a sterile container, and an antibiotic supplement.

Brief Description of the Figures

Figure 1. Effect of AII on human neuronal progenitor neurite outgrowth.

Figure 2. Effect of AII, AII(1-7), and Ala4-AIII on the proliferation of normal human neural progenitors.

Detailed Description of the Preferred Embodiments

As defined herein, the term "neuronal cells" include either primary cells or established cell lines with the potential to differentiate into neurons, astrocytes, and oligodendrocytes and to self renew, and also to differentiated cells derived therefrom, including fully differentiated CNS and peripheral nervous system ("PNS") cell types. Examples of neuronal stem and progenitor cells include, but are not limited to, those described in Gritti et al., *J. of Neuroscience* 16:1091-1100 (1996); Frederiksen et al., (1988); Reynolds and Weiss, (1992); Davis and Temple, (1994); McKay, (1997);. Vicario-Abejon et al., (1995); Craig et al., (1996); Tontsch et al. (1994); Graves et al., (1993) and Johe et al., *Genes and Develop.* 10:3129-3142 (1996), all references hereby incorporated in their entirety. As defined herein, "proliferation" encompasses both cellular self renewal and cellular proliferation with accompanying differentiation.

Unless otherwise indicated, the term "active agents" as used herein refers to the group of compounds comprising angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II (AII), AII analogues, AII fragments or analogues thereof and AII AT_2 type 2 receptor agonists.

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991, Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX).

U.S. Patent No. 5,015,629 to DiZerega (the entire disclosure of which is hereby incorporated by reference) describes a method for increasing the rate of healing of wound tissue, comprising the application to such tissue of angiotensin II (AII) in an amount which is sufficient for said increase. The application of AII to wound tissue significantly increases the rate of wound healing, leading to a more rapid re-epithelialization and tissue repair. The term AII refers to an octapeptide present in humans and other species having the sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:1]. The biological formation of angiotensin is initiated by the action of renin on the plasma substrate angiotensinogen. The substance so formed is a decapeptide called angiotensin I (AI) which is converted to AII by the converting enzyme angiotensinase which removes the C-terminal His-Leu residues from AI (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu [SEQ ID NO:37]). AII is a known pressor agent and is commercially available. The use of AII analogues and fragments, AT2 agonists, as well as AIII and AIII analogues and fragments in wound healing has also been described. (U.S.

Patent No. 5,629,292; U.S. Patent No. 5,716,935; WO 96/39164; all references herein incorporated by reference in their entirety.)

Studies have shown that AII increases mitogenesis and chemotaxis in cultured cells that are involved in wound repair, and also increases their release of growth factors and extracellular matrices (diZerega, U.S. Patent No. 5,015,629; Dzau et. al., *J. Mol. Cell. Cardiol.* 21:S7 (Supp III) 1989; Berk et. al., *Hypertension* 13:305-14 (1989); Kawahara, et al., *BBRC* 150:52-9 (1988); Naftilan, et al., *J. Clin. Invest.* 83:1419-23 (1989); Taubman et al., *J. Biol. Chem* 264:526-530 (1989); Nakahara, et al., *BBRC* 184:811-8 (1992); Stouffer and Owens, *Circ. Res.* 70:820 (1992); Wolf, et al., *Am. J. Pathol.* 140:95-107 (1992); Bell and Madri, *Am. J. Pathol.* 137:7-12 (1990). In addition, AII was shown to be angiogenic in rabbit corneal eye and chick chorioallantoic membrane models (Fernandez, et al., *J. Lab. Clin. Med.* 105:141 (1985); LeNoble, et al., *Eur. J. Pharmacol.* 195:305-6 (1991). Therefore, AII may accelerate wound repair through increased neovascularization, growth factor release, reepithelialization and/or production of extracellular matrix.

AII has also been implicated in both cell growth and differentiation (Meffert et al., *Mol. and Cellul. Endocrin.* 122:59 (1996)). Two main classes of AII receptors, AT_1 and AT_2 have been identified (Meffert, 1996). The growth-promoting effects of AII have been attributed to mediation by the AT1 receptor, while some evidence suggests that the AT2 receptor may be involved in mediation of the cell differentiation effects of AII (Bedecs et al., *Biochem. J.* 325:449 (1997)).

The effects of AII receptor and AII receptor antagonists have been examined in two experimental models of vascular injury and repair which suggest that both AII receptor subtypes (AT1 and AT2) play a role in wound healing (Janiak et al., *Hypertension* 20:737-45 (1992); Prescott, et al., *Am. J. Pathol.* 139:1291-1296 (1991); Kauffman, et al., *Life Sci.*

49:223-228 (1991); Viswanathan, et al., *Peptides* 13:783-786 (1992); Kimura, et al., *BBRC* 187:1083-1090 (1992).

Many studies have focused upon AII(1-7) (AII residues 1-7) or other fragments of AII to evaluate their activity. AII(1-7) elicits some, but not the full range of effects elicited by AII. Pfeilschifter, et al., *Eur. J. Pharmacol.* 225:57-62 (1992); Jaiswal, et al., *Hypertension* 19(Supp. II):II-49-II-55 (1992); Edwards and Stack, *J. Pharmacol. Exper. Ther.* 266:506-510 (1993); Jaiswal, et al., *J. Pharmacol. Exper. Ther.* 265:664-673 (1991); Jaiswal, et al., *Hypertension* 17:1115-1120 (1991); Portsi, et a., *Br. J. Pharmacol.* 111:652-654 (1994).

Studies have shown that AII inhibits proliferation of both an immortalized neuronal cell line (the pheochromocytoma derived PC12W; Meffert, 1996), and of dissociated primary cultures of retrochiamatic hypothalamus from 18-day old rat embryos (Jirikowski et al., *Develop. Brain Res.* 14:179-183 (1984)). Other studies have shown that the proportion of AT_1 and AT_2 receptors in rat brain changes during development, with fetal tissue expressing far more AT_2 receptor subtype, while adult animals express far more AT_1 subtype (Meffert, 1996). However, little is known about AII effects on most CNS or peripheral nervous system ("PNS") cell types. Furthermore, it is not known what AII receptor subtypes are expressed by neuronal stem and progenitor cells, nor what effect AII has on their proliferative capacity.

A peptide agonist selective for the AT2 receptor (AII has 100 times higher affinity for AT2 than AT1) is p-aminophenylalanine6-AII ["(p-NH_2-Phe)6-AII)"], Asp-Arg-Val-Tyr-Ile-Xaa-Pro-Phe [SEQ ID NO.36] wherein Xaa is p-NH_2-Phe (Speth and Kim, *BBRC* 169:997-1006 (1990). This peptide gave binding characteristics comparable to AT2 antagonists in the experimental models tested (Catalioto, et al., *Eur. J. Pharmacol.* 256:93-97 (1994); Bryson, et al., *Eur. J. Pharmacol.* 225:119-127 (1992).

The active AI, AI analogues, AI fragments and analogues thereof, AII analogues, fragments of AII and analogues thereof of particular interest in accordance with the present invention are characterized as comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group,

R^A is suitably selected from Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc,

R^B is suitably selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys, and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO_3)$_2$, Thr, Ser, homoSer, Ala, and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group.

Compounds falling within the category of AT2 agonists useful in the practice of the invention include the AII analogues set forth above subject to the restriction that R^6 is p-NH_2-

Phe. In addition to peptide agents, various nonpeptidic agents (e.g., peptidomimetics) having the requisite AT2 agonist activity are further contemplated for use in accordance with the present invention.

Particularly preferred combinations for R^A and R^B are Asp-Arg, Asp-Lys, Glu-Arg and Glu-Lys. Particularly preferred embodiments of this class include the following: AII, AIII or AII(2-8), Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]; AII(3-8), also known as des1-AIII or AIV, Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:3]; AII(1-7), Asp-Arg-Val-Tyr-Ile-His-Pro [SEQ ID NO:4]; AII(2-7). Arg-Val-Tyr-Ile-His-Pro [SEQ ID NO:5]; AII(3-7), Val-Tyr-Ile-His-Pro [SEQ ID NO:6]; AII(5-8), Ile-His-Pro-Phe [SEQ ID NO:7]; AII(1-6), Asp-Arg-Val-Tyr-Ile-His [SEQ ID NO:8]; AII(1-5), Asp-Arg-Val-Tyr-Ile [SEQ ID NO:9]; AII(1-4), Asp-Arg-Val-Tyr [SEQ ID NO:10]; and AII(1-3), Asp-Arg-Val [SEQ ID NO:11]. Other preferred embodiments include: Arg-norLeu-Tyr-Ile-His-Pro-Phe [SEQ ID NO:12] and Arg-Val-Tyr-norLeu-His-Pro-Phe [SEQ ID NO:13]. Still another preferred embodiment encompassed within the scope of the invention is a peptide having the sequence Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe [SEQ ID NO:31]. AII(6-8), His-Pro-Phe [SEQ ID NO:14] and AII(4-8), Tyr-Ile-His-Pro-Phe [SEQ ID NO:15] were also tested and found not to be effective.

In another preferred embodiment, the present invention provides a method for promoting neuronal cell proliferation or differentiation comprising contacting neuronal cells with an amount effective to promote proliferation or differentiation of at least one active agent comprising a sequence consisting of the general formula:

R1-ARG-VAL-TYR-R2-HIS-PRO-R3

wherein R1 is selected from the group consisting of H or Asp;

R2 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R3 is either Phe or H.

In a most preferred embodiment, the active agent is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:26, and SEQ ID NO:34.

Another class of compounds of particular interest in accordance with the present invention are those of the general formula II

$$R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^2 is selected from the group consisting of H, Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr$(PO_3)_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr.

A particularly preferred subclass of the compounds of general formula II has the formula

$$R^2\text{-}R^3\text{-Tyr-}R^5\text{-His-Pro-Phe}$$ [SEQ ID NO:16]

wherein R^2, R^3 and R^5 are as previously defined. Particularly preferred is angiotensin III of the formula Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]. Other preferred compounds include peptides having the structures Arg-Val-Tyr-Gly-His-Pro-Phe [SEQ ID NO:17] and

Arg-Val-Tyr-Ala-His-Pro-Phe [SEQ ID NO:18]. The fragment AII(4-8) was ineffective in repeated tests; this is believed to be due to the exposed tyrosine on the N-terminus.

In the above formulas, the standard three-letter abbreviations for amino acid residues are employed. In the absence of an indication to the contrary, the L-form of the amino acid is intended. Other residues are abbreviated as follows:

TABLE 1

Abbreviation for Amino Acids

Me^2Glv	N,N-dimethylglycyl
Bet	1-carboxy-N,N,N-trimethylmethanaminium hydroxide inner salt (betaine)
Suc	Succinyl
Phe(Br)	p-bromo-L-phenylalanyl
azaTyr	aza-α'-homo-L-tyrosyl
Acpc	1-aminocyclopentane carboxylic acid
Aib	2-aminoisobutyric acid
Sar	N-methylglycyl (sarcosine)

It has been suggested that AII and its analogues adopt either a *gamma* or a *beta* turn (Regoli, et al., *Pharmacological Reviews* 26:69 (1974). In general, it is believed that neutral side chains in position R^3, R^5 and R^7 may be involved in maintaining the appropriate distance between active groups in positions R^4, R^6 and R^8 primarily responsible for binding to receptors and/or intrinsic activity. Hydrophobic side chains in positions R^3, R^5 and R^8 may also play an important role in the whole conformation of the peptide and/or contribute to the formation of a hypothetical hydrophobic pocket.

Appropriate side chains on the amino acid in position R^2 may contribute to affinity of the compounds for target receptors and/or play an important role in the conformation of the peptide. For this reason, Arg and Lys are particularly preferred as R^2.

For purposes of the present invention, it is believed that R^3 may be involved in the formation of linear or nonlinear hydrogen bonds with R^5 (in the gamma turn model) or R^6 (in the beta turn model). R^3 would also participate in the first turn in a beta antiparallel structure (which has also been proposed as a possible structure). In contrast to other positions in general formula I, it appears that beta and gamma branching are equally effective in this position. Moreover, a single hydrogen bond may be sufficient to maintain a relatively stable conformation. Accordingly, R^3 may suitably be selected from Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr. In another preferred embodiment, R^3 is Lys.

With respect to R^4, conformational analyses have suggested that the side chain in this position (as well as in R^3 and R^5) contribute to a hydrophobic cluster believed to be essential for occupation and stimulation of receptors. Thus, R^4 is preferably selected from Tyr, Thr, Tyr $(PO_3)_2$, homoSer, Ser and azaTyr. In this position, Tyr is particularly preferred as it may form a hydrogen bond with the receptor site capable of accepting a hydrogen from the phenolic hydroxyl (Regoli, et al. (1974), *supra*). In a further preferred embodiment, R^4 is Ala.

In position R^5, an amino acid with a β aliphatic or alicyclic chain is particularly desirable. Therefore, while Gly is suitable in position R^5, it is preferred that the amino acid in this position be selected from Ile, Ala, Leu, norLeu, Gly and Val.

In the AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, fragments and analogues of fragments of particular interest in accordance with the present invention, R^6 is His, Arg or 6-NH_2-Phe. The unique properties of the imidazole ring of histidine (e.g., ionization at physiological pH, ability to act as proton donor or acceptor,

aromatic character) are believed to contribute to its particular utility as R^6. For example, conformational models suggest that His may participate in hydrogen bond formation (in the *beta* model) or in the second turn of the antiparallel structure by influencing the orientation of R^7. Similarly, it is presently considered that R^7 should be Pro in order to provide the most desirable orientation of R^8. In position R^8, both a hydrophobic ring and an anionic carboxyl terminal appear to be particularly useful in binding of the analogues of interest to receptors; therefore, Tyr and especially Phe are preferred for purposes of the present invention.

Analogues of particular interest include the following:

TABLE 2

Angiotensin II Analogues

AII Analogue Name	Amino Acid Sequence	Sequence Identifier
Analogue 1	Asp-Arg-Val-Tyr-Val-His-Pro-Phe	SEQ ID NO: 19
Analogue 2	Asn-Arg-Val-Tyr-Val-His-Pro-Phe	SEQ ID NO: 20
Analogue 3	Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe	SEQ ID NO: 21
Analogue 4	Glu-Arg-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 22
Analogue 5	Asp-Lys-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 23
Analogue 6	Asp-Arg-Ala-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 24
Analogue 7	Asp-Arg-Val-Thr-Ile-His-Pro-Phe	SEQ ID NO: 25
Analogue 8	Asp-Arg-Val-Tyr-Leu-His-Pro-Phe	SEQ ID NO: 26
Analogue 9	Asp-Arg-Val-Tyr-Ile-Arg-Pro-Phe	SEQ ID NO: 27
Analogue 10	Asp-Arg-Val-Tyr-Ile-His-Ala-Phe	SEQ ID NO: 28
Analogue 11	Asp-Arg-Val-Tyr-Ile-His-Pro-Tyr	SEQ ID NO: 29
Analogue 12	Pro-Arg-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 30
Analogue 13	Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 31
Analogue 14	Asp-Arg-Val-Tyr$(PO_3)_2$-Ile-His-Pro-Phe	SEQ ID NO: 32
Analogue 15	Asp-Arg-norLeu-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 33
Analogue 16	Asp-Arg-Val-Tyr-norLeu-His-Pro-Phe	SEQ ID NO: 34
Analogue 17	Asp-Arg-Val-homoSer-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 35

The polypeptides of the instant invention may be synthesized by methods such as those set forth in J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, 2nd ed., Pierce Chemical Co., Rockford, Ill. (1984) and J. Meienhofer, *Hormonal Proteins and*

Peptides. Vol. 2, Academic Press, New York, (1973) for solid phase synthesis and E. Schroder and K. Lubke, *The Peptides*, Vol. 1, Academic Press, New York, (1965) for solution synthesis. The disclosures of the foregoing treatises are incorporated by reference herein.

In general, these methods involve the sequential addition of protected amino acids to a growing peptide chain (U.S. Patent No. 5,693,616, herein incorporated by reference in its entirety). Normally, either the amino or carboxyl group of the first amino acid and any reactive side chain group are protected. This protected amino acid is then either attached to an inert solid support, or utilized in solution, and the next amino acid in the sequence, also suitably protected, is added under conditions amenable to formation of the amide linkage. After all the desired amino acids have been linked in the proper sequence, protecting groups and any solid support are removed to afford the crude polypeptide. The polypeptide is desalted and purified, preferably chromatographically, to yield the final product.

In one aspect of the present invention, a method of increasing *in vivo*, *in vitro* and *ex vivo* neuronal stem and progenitor cell proliferation by exposure to angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists ("active agents") is disclosed. Experimental conditions for the isolation, purification, *in vitro*/*ex vivo* growth and *in vivo* mobilization of neuronal stem and progenitor cells have been reported (Frederiksen et al., 1988; Reynolds and Weiss, 1992; Gritti et al., 1996; Vicario-Abejon et al., 1995; Johe et al., 1996; Craig et al., 1996; Suhonen et al., *Nature* 383:624-627, 1996; and Tontsch et al., 1994).

Proliferation can be quantitated using any one of a variety of techniques well known in the art, including, but not limited to, bromodeoxyuridine incorporation (Vicario-Abejon et al., 1995), ^{3}H-thymidine incorporation (Fredericksen et al., 1988), or antibody labeling of a protein present in higher concentration in proliferating cells than in non-proliferating cells. In

a preferred embodiment, proliferation of neuronal stem and progenitor cells is assessed by reactivity to an antibody directed against a protein known to be present in higher concentrations in proliferating cells than in non-proliferating cells, including but not limited to proliferating cell nuclear antigen (PCNA, or cyclin; Zymed Laboratories, S uth San Francisco, California).

In one embodiment, neuronal cells are isolated from primary cell masses according to standard methods (Jirikowski et al., 1984; Reynolds and Weiss, 1992; Johe et al., 1996; Gritti et al., 1996; Vicario-Abejon et al., 1995; Kordower et al., 1995; Nauert and Freeman, *Cell Transplant* 3:147-151, 1994; Freeman and Kordower, In: Lindvall et al., eds. *Intracerebral Transplantation in Movement Disorders*. New York: Elsevier Science, 163-170, 1991), suspended in culture medium and incubated in the presence of, preferably, between about 0.1 ng/ml and about 10 mg/ml of the active agents of the invention. The cells are expanded for a period of between 8 and 21 days and cellular proliferation is assessed as described above.

In a preferred embodiment, neuronal stem and progenitor cells are isolated from primary cells which are isolated from the adult rat mammalian forebrain or rat embryonic hippocampus (Johe et al., 1996). The cell mass is dissociated by either mechanical trituration or by incubating minced tissue in Hank's Buffered Saline Solution (HBSS). The cells are collected by centrifugation and resuspended in a serum-free medium containing Dulbecco's modified Eagle medium (DMEM)/F12, glucose, glutamine, sodium bicarbonate, 25 µg/ml of insulin, 100 µg/ml of human apotransferrin, 20 nm progesterone, 100 µm putrescene, 30 nm sodium selenite (pH 7.2), plus 10 ng/ml of recombinant basic fibroblast growth factor (bFGF; R&D, Inc.) (Johe et al., 1996). The cells are plated into tissue culture plates precoated with cell attachment factors, as is well known in the art. BFGF is added daily, and the medium is

changed every two days. Cells are passaged at 50% confluence by briefly incubating them in HBSS and scraping with a cell scraper.

Alternatively, neuronal stem and progenitor cells are isolated from the dissociated cell mass by antibody-mediated cell capture ("panning"; Barres et al., Cell 70:31-46, 1992). Antibodies that can be used to isolate neuronal stem and precursor cells include, but are not limited to, nestin antibody (Vicario-Abejon et al., 1995). The cells are then treated as above.

The neuronal stem and progenitor cells exposed to the active agents as described above can be used for neuron replacement therapy, to treat disorders including, but not limited to, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis. The cells are cultured *in vitro* or *ex vivo* as described above. The cells are rinsed to remove all traces of culture fluid, resuspended in an appropriate medium and then pelleted and rinsed several times. After the final rinse, the cells are resuspended at between 0.7×10^6 and 50×10^6 cells per ml in an appropriate medium and used for transplantation according to previously described methods. (Kordower et al., 1995; Freed et al., *N. Engl. J. Med.* 327:1549-1555, 1992; *Ann. Neurol.* 31:155-165, 1992; Peschanski et al., *Brain* 117:487-499, 1994; Spencer et al., *N. Engl. J. Med.* 327:1541-1548, 1992; Henderson et al., *Arch. Neurol.* 48:822-827, 1992; Hitchcock et al., *Exp. Neurol.* 129:3, 1994; Lindvall et al., *Science* 247:574-577, 1990; Widner et al., *N. Engl. J. Med.* 327:1556-1563, 1992; Bankiewicz et al., *J. Neurosurg.* 72:231-244, 1990; Kordower et al., *Ann. Neurol.* 29:405-412, 1991)

In a preferred embodiment, the neuronal stem and progenitor cells used for transplantation are transfected with an expression vector so as to express a therapeutic protein, including but not limited to glial-cell-line-derived neurotrophic factor (GDNF; Beck et al. *Nature* 373:339-341, 1995; Tomac et al., *Nature* 373:335-339, 1995) after transplantation.

In a further aspect of the present invention, the effect of the active agents on neuronal stem and progenitor cell differentiation is assessed by examination of changes in gene expression, phenotype, morphology, or any other method that distinguishes stem and/or progenitor cells from fully differentiated cells. Examples of such differentiation markers against which antibodies are available include, but are not limited to, neuron-specific microtubule-associated protein 2 (MAP2; Vicario-Abejon et al., 1995; antibody available from Boehringer Manheim, Germany), astroglial-specific glial fibrillary acidic protein (GFAP; Vicario-Abejon et al., 1995; antibody available from Incstar,); neuron-specific tau protein (Johe et al., 1996; antibody available from Sigma, St. Louis, MO); neurofilaments L and M (Johe et al., 1996; antibody available from Boehringer Manheim); and oligodendrocyte-specific O4 and galactocerebroside (GalC; Johe et al., 1996). The DNA sequences for all of these differentiation-specific markers are known, and thus PCR amplification and/or hybridization studies to evaluate gene expression of the differentiation markers can be performed according to standard methods in the art. (Johe et al., 1996)

In a preferred embodiment, neuronal stem and progenitor cells are isolated and cultured as described above. Differentiation is initiated by contacting the cells with the active agents as described above, in serum-free medium in the absence of bFGF. Differentiation is assessed at various times by immunodetection of differentiation-specific markers, using the antibodies described above (Johe et al., 1996). Alternatively, differentiation is assessed morphologically by measurement of neurite outgrowth.

In another aspect of the present invention the active agents are used to increase *in vivo* neuronal stem and progenitor cell proliferation. For use in increasing proliferation of neuronal stem and progenitor cells, the active agents may be administered by any suitable route, including orally, parentally, by inhalation spray, rectally, or topically in dosage unit

formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. The term parenteral as used herein includes, subcutaneous, intravenous, intra-arterial, intra-ventricular, intramuscular, intrasternal, intratendinous, intraspinal, intracranial, intrathoracic, infusion techniques or intraperitoneally.

The active agents may be made up in a solid form (including granules, powders or suppositories) or in a liquid form (*e.g.*, solutions, suspensions, or emulsions) and may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc.

While the active agents can be administered as the sole active agent, they can also be used in combination with one or more other compounds. When administered as a combination, the active agents and other compounds can be formulated as separate compositions that are given at the same time or different times, or the active agents and other compounds can be given as a single composition.

For administration, active agents are ordinarily combined with one or more adjuvants appropriate for the indicated route of administration. The compounds may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinylpyrrolidine, and/or polyvinyl alcohol, and tableted or encapsulated for conventional administration. Alternatively, the compounds of this invention may be dissolved in saline, water, polyethylene glycol, propylene glycol, carboxymethyl cellulose colloidal solutions, ethanol, corn oil, peanut oil, cottonseed oil, sesame oil, tragacanth gum, and/or various buffers. Other adjuvants and modes of administration are well known in the pharmaceutical art. The carrier or diluent may include

time delay material, such as glyceryl monostearate or glyceryl distearate alone or with a wax, or other materials well known in the art.

Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin (*e.g.*, liniments, lotions, ointments, creams, or pastes) and drops suitable for administration to the eye, ear, or nose.

The dosage regimen for increasing *in vivo* proliferation or differentiation of neuronal stem and progenitor cell with the active agents of the invention is based on a variety of factors, including the type of injury or deficiency, the age, weight, sex, medical condition of the individual, the severity of the condition, the route of administration, and the particular compound employed. Thus, the dosage regimen may vary widely, but can be determined routinely by a physician using standard methods. Dosage levels of the order of between 0.1 ng/kg and 10 mg/kg angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and/or AII AT_2 type 2 receptor agonists per body weight are useful for all methods of use disclosed herein.

In a preferred embodiment of the present invention, the active agents are administered by unilateral infusion directly into the mammalian brain lateral ventricle using an osmotic pump (Alza Palo Alto, CA) attached to a 30 gauge cannulae implanted at the injection coordinate, as described in Craig et al., J. of Neuroscience 16:2649-2658 (1996). A suitable injected dose of active ingredient of the active agents is preferably between about 0.1 ng/kg and about 10 mg/kg administered twice daily. The active ingredient may comprise from 0.001% to 10% w/w, *e.g.*, from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w, but preferably not more than 5% w/w, and more preferably from 0.1% to 1% of the formulation.

In another aspect of the present invention, an improved cell culture medium is provided for the proliferation and differentiation of neuronal cells, wherein the improvement comprises addition to the cell culture medium of an effective amount of the active agents, as described above. Any cell culture media that can support the growth of neuronal stem and progenitor cells can be used with the present invention. Such cell culture media include, but are not limited to Basal Media Eagle, Dulbecco's Modified Eagle Medium, Iscove's Modified Dulbecco's Medium, McCoy's Medium, Minimum Essential Medium, F-10 Nutrient Mixtures, Opti-MEM® Reduced-Serum Medium, RPMI Medium, and Macrophage-SFM Medium or combinations thereof.

The improved cell culture medium can be supplied in either a concentrated (ie: 10X) or non-concentrated form, and may be supplied as either a liquid, a powder, or a lyophilizate. The cell culture may be either chemically defined, or may contain a serum supplement. Culture media is commercially available from many sources, such as GIBCO BRL (Gaithersburg, MD) and Sigma (St. Louis, MO)

In a further aspect, the present invention provides kits for the propagation of neuronal stem and progenitor cells, wherein the kits comprise an effective amount of the active agents, as described above.

In a preferred embodiment, the kit further comprises cell culture growth medium. Any cell culture media that can support the growth of neuronal stem and progenitor cells can be used with the present invention. Examples of such cell culture media are described above.

The improved cell culture medium can be supplied in either a concentrated (ie: 10X) or non-concentrated form, and may be supplied as either a liquid, a powder, or a lyophilizate. The cell culture may be either chemically defined, or may contain a serum supplement.

In another preferred embodiment, the kit further comprises a sterile container. The sterile container can comprise either a sealed container, such as a cell culture flask, a roller bottle, or a centrifuge tube, or a non-sealed container, such as a cell culture plate or microtiter plate (Nunc; Naperville, IL).

In a further preferred embodiment, the kit further comprises an antibiotic supplement for inclusion in the reconstituted cell growth medium. Examples of appropriate antibiotic supplements include, but are not limited to actimonycin D, Fungizone®, kanamycin, neomycin, nystatin, penicillin, streptomycin, or combinations thereof (GIBCO).

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined by the claims appended hereto.

Example 1. AII Effect on the Proliferation and Differentiation of Normal Human Neuronal Progenitor Cells

Normal human progenitor cells were purchased from Clonetics (San Diego, CA) and cultured in Neural Progenitor Cell Maintenance Medium (NPMM) (Neural Progenitor Basal Medium containing human recombinant fibroblast growth factor beta, human recombinant epidermal growth factor, neural survival factors, gentamycin and amphotericin B). The cells were thawed, diluted into NPMM and cultured for 24 hours in a 75 cm^2 flask. The cells were cultured in dedifferentiaited spheroids until studies were conducted to assess differentiation. When the cells were cultured in suspension culture in the presence of 1, 10, or 100 µg/ml AII for 4-7 days prior to placement of the cells on a culture substrate that allowed adherence and differentiation (as described further below), an increase in the number of cells able to undergo differentiation (ie: proliferation) was observed (Table I).

Table I. Effect of AII on the Proliferation of Human Neuronal Progenitor Cells

AII Concentration	Cells per Well on Day 4	Cells per Well on Day 7
0 µg/ml	66	63
1 µg/ml	93	85
10 µg/ml	89	86
100 µg/ml	91	86

Assessment of Differentiation

In order to assess the differentiation of neuronal cells, the cells were seeded upon wells coated with 0.05% polyethyleneimine (PEI) substrate in borate buffer solution. The wells of a 96 well plate were coated with 0.05 ml of this solution overnight at room temperature. After the incubation, the substrate was removed by aspiration, rinsed with sterile water and allowed to dry before seeding of the cells.

After culture of cells for 4-7 days in the presence of AII (to assess proliferation), the cells were washed and placed in PEI-coated wells to assess differentiation. Four days after plating, the number of cells undergoing differentiation, as assessed by neurite outgrowth, was counted (see Table 1).

In an additional study, the effect of AII on the differentiation of neuronal progenitor cells was assessed. After adherence to PEI substrate, the cells cease proliferation and undergo differentiation and neurite outgrowth. One thousand cells were placed in each well in the presence and absence of various concentrations of AII. Four and seven days after initiation of culture, the size of the neurites (by measurement with an ocular micrometer) on the cells undergoing differentiation and the number of cells undergoing differentiation was assessed. Culture of the cells in the presence of AII increased the rate of neurite outgrowth (see Figure 1) and the number of cells undergoing differentiation by approximately 50% (data not shown).

These studies demonstrate that exposure to AII promotes the proliferation and differentiation of normal human neuronal progenitor cells.

Example 2. *Effect of AII, AII(1-7), and ALa4-AIII on the proliferation of normal human neural progenitors*

Normal human progenitor cells were purchased from Clonetics (San Diego, CA) and cultured in Neuronal Progenitor Cell Maintenance Medium (NPMM) (Neural Progenitor Basal Medium containing human recombinant fibroblast growth factor beta, human recombinant epidermal growth factor, neural survival factors, gentamycin, and amphotericin B). The cells were thawed, diluted into NPMM and cultured for 24 hours in a 75 cm^2 flask. Until studies to assess differentiation, the cells were cultured in dedifferentiated spheroids. If the cells were cultured in suspension culture in the presence of 10 μg/ml AII (SEQ ID NO:1), AII(1-7) (SEQ ID NO:4), or Ala4-AIII (SEQ ID NO:18) for 7 days prior to placement of the cells on collagen-coated plates to allow adherence, an increase in the number of cells able to undergo proliferation was observed (Figure 2). The increase in the number of human neural progenitors in the wells was 300% in the presence of AII, 175% in the presence of Ala4-AIII, and 100% in the presence of AII(1-7), while the increase was only 33% in the control wells. These studies show that each of these peptides promoted the proliferation of normal human neuronal progenitor cells.

The present invention, by providing a method for enhanced proliferation of neuronal cells, will greatly increase the clinical benefits of neuronal stem and progenitor transplantation. This is true both for increased "self-renewal" of neuronal stem cells, which will provide a larger supply of stem cells capable of differentiation into various neuronal cell types, and for proliferation with differentiation, which will provide a larger supply of neuronal progenitor and differentiated cells at the appropriate site. Similarly, methods that increase *in*

vivo proliferation of neuronal stem, progenitor, and differentiated cells are beneficial in treating many neurological disorders, including Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis.

The method of the present invention also increases the potential utility of neuronal stem and progenitor cells as vehicles for gene therapy in central and peripheral nervous system disorders by more efficiently providing a large number of such cells for transfection, and also by providing a more efficient means to rapidly expand transfected neuronal stem and progenitor cells.

The present invention is not limited by the aforementioned particular preferred embodiments. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed preferred embodiments without diverting from the concept of the invention. All such modifications are intended to be within the scope of the present invention.

We claim:

1. A method for promoting neuronal cell proliferation or differentiation comprising contacting neuronal cells with an amount effective to promote proliferation or differentiation of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group

R^A is selected from Asp, Glu, Asn, Acpc, Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc;

R^B is selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys, and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO_3)$_2$, Thr, Ser, homoSer, Ala, and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group.

2. The method of claim 1 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO. 1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID

NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36; and SEQ ID NO:37.

3. The method of claim 1 wherein the active agent is SEQ ID NO:1, SEQ ID NO:4, or SEQ ID NO:18.

4. The method of claim 1 wherein the concentration of active agent is between about 0.1 ng/kg and about 10.0 mg/kg.

5. An improved cell culture medium for promotion of neuronal cell proliferation or differentiation, wherein the improvement comprises addition to the cell culture medium an amount effective to increase proliferation or differentiation of neuronal cells of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group

R^A is selected from Asp, Glu, Asn, Acpc, Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc;

R^B is selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys, and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr$(PO_3)_2$, Thr, Ser, homoSer, Ala, and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group.

6. The improved cell culture medium of claim 5 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO. 1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, and SEQ ID NO:37.

7. The improved cell culture of claim 5 wherein the active agent is SEQ ID NO:1, SEQ ID NO:4, or SEQ ID NO:18.

8. The improved cell culture medium of claim 5 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

9. A kit for promoting neuronal cell proliferation or differentiation comprising:

(a) an amount effective to promote neuronal cell proliferation or differentiation of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group

R^A is selected from Asp, Glu, Asn, Acpc, Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc;

R^B is selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys, and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO_3)$_2$, Thr, Ser, homoSer, Ala, and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group; and

(b) instructions for using the amount effective of active agent to promote neuronal cell proliferation or differentiation.

10. The kit of claim 9 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO. 1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29,

SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, and SEQ ID NO:37.

11. The kit of claim 9 wherein the active agent is SEQ ID NO:1, SEQ ID NO:4, or SEQ ID NO:18.

12. The kit of claim 9 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

13. A method for promoting neuronal cell proliferation or differentiation comprising contacting neuronal cells with an amount effective to promote proliferation or differentiation of at least one active agent comprising a sequence consisting of the general formula:

R1-ARG-VAL-TYR-R2-HIS-PRO-R3

wherein R1 is selected from the group consisting of H or Asp;

R2 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R3 is either Phe or H.

14. The method of claim 13 wherein the active agent is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:26, and SEQ ID NO:34.

15. The method of claim 13 wherein the concentration of active agent is between about 0.1 ng/kg and about 10.0 mg/kg.

16. An improved cell culture medium for promotion of neuronal cell proliferation or differentiation, wherein the improvement comprises addition to the cell culture medium an amount effective to increase proliferation or differentiation of neuronal cells of at least one active agent comprising a sequence consisting of the general formula I

R1-ARG-VAL-TYR-R2-HIS-PRO-R3

wherein R1 is selected from the group consisting of H or Asp;

R2 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R3 is either Phe or H.

17. The improved cell culture medium of claim 16 wherein the active agent is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:26, and SEQ ID NO:34.

18. The improved cell culture medium of claim 16 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

19. A kit for promoting neuronal cell proliferation or differentiation comprising:

(a) an amount effective to promote neuronal cell proliferation or differentiation of at least one active agent comprising a sequence consisting of the general formula:

R1-ARG-VAL-TYR-R2-HIS-PRO-R3

wherein R1 is selected from the group consisting of H or Asp;

R2 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R3 is either Phe or H; and

(b) instructions for using the amount effective of active agent to promote neuronal cell proliferation or differentiation.

20. The kit of claim 18 wherein the active agent is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:26, and SEQ ID NO:34.

21. The kit of claim 18 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

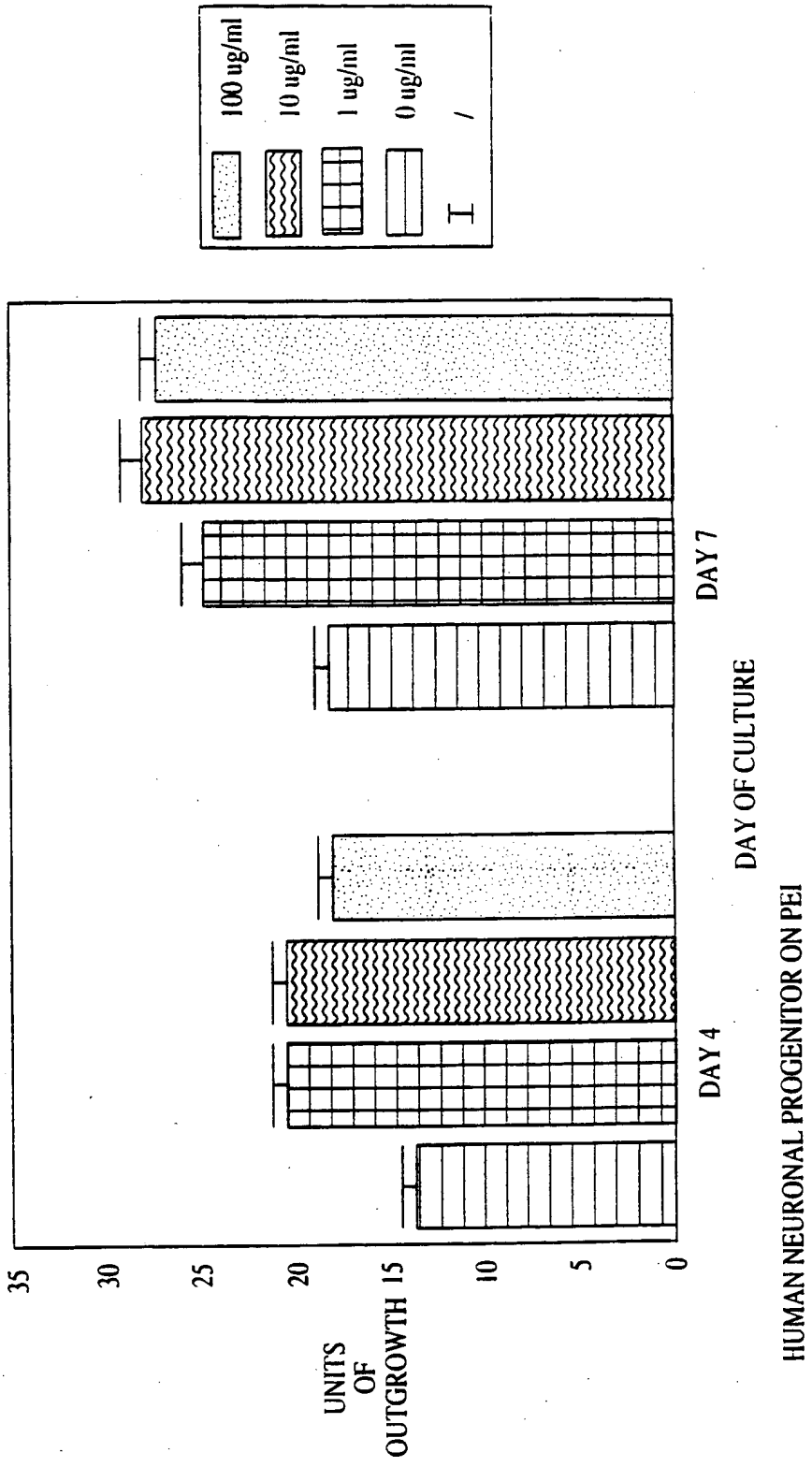
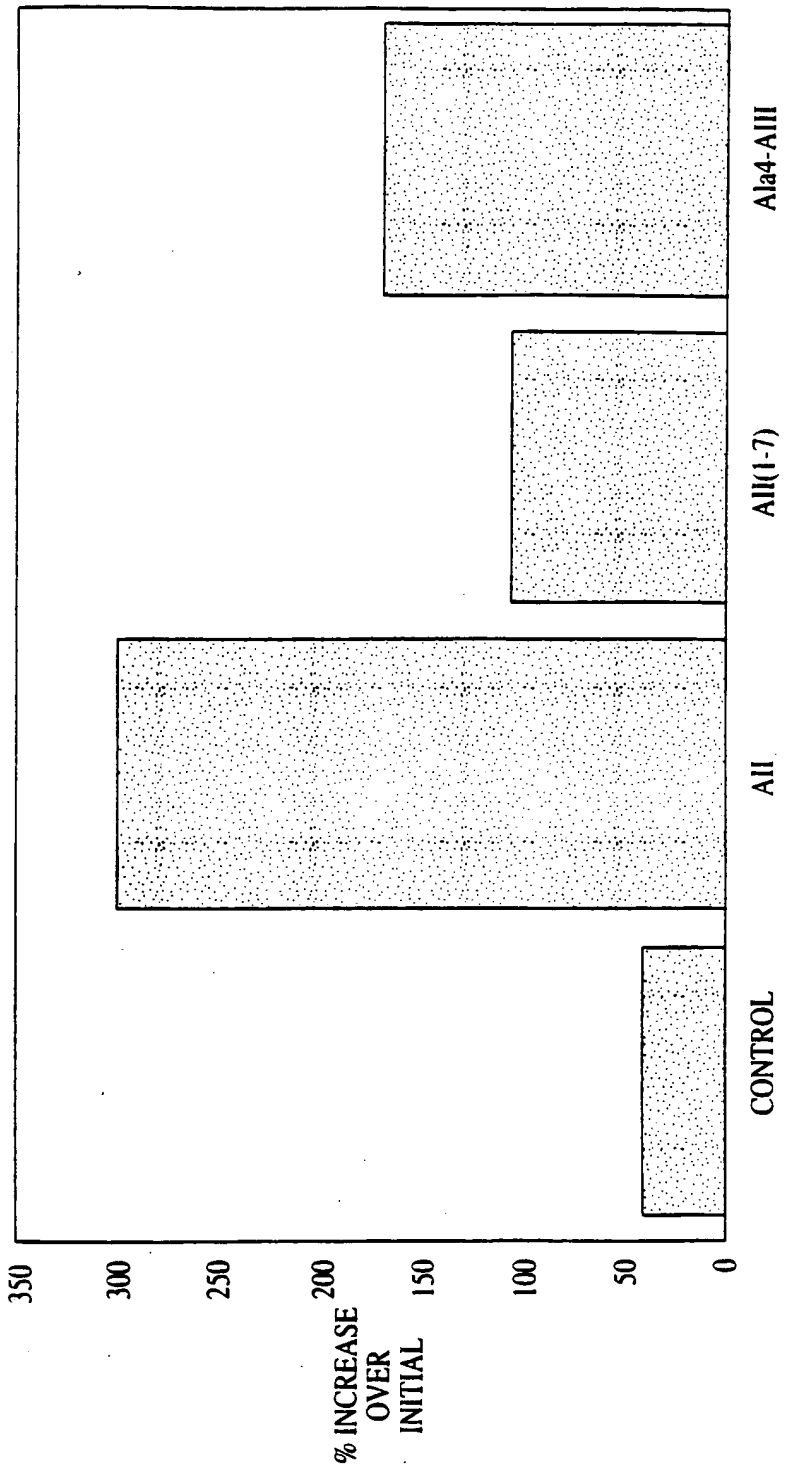

100 ug/ml
10 ug/ml
1 ug/ml
0 ug/ml
35
30
25
20
15
10
5
0
UNITS OF OUTGROWTH
DAY 4
DAY 7
DAY OF CULTURE
HUMAN NEURONAL PROGENITOR ON PEI

FIG. 1


350
300
250
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% INCREASE OVER INITIAL
INCREASE IN CELL NUMBER OVER INITIAL PLATED
CONTROL
AII
AII(1-7)
Ala4-AIII
PEPTIDE TESTED

FIG. 2

SEQUENCE LISTING

<210> 1

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII

<400> 1

Asp Arg Val Tyr Ile His Pro Phe

1 5

<210> 2

<211> 7

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII (2-8)

<400> 2

Arg Val Tyr Ile His Pro Phe

1 5

<210> 3

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII (3-8)

<400> 3

Val Tyr Ile His Pro Phe

1 5

<210> 4

<211> 7

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII (1-7)

<400> 4

Asp Arg Val Tyr Ile His Pro

1 5

<210> 5

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII (2-7)

<400> 5

Arg Val Tyr Ile His Pro

1 5

<210> 6

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII (3-7)

<400> 6

Val Tyr Ile His Pro

1 5

<210> 7

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII (5-8)

<400> 7

Ile His Pro Phe

1

<210> 8

<211> 6

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence:AII (1-6)

<400> 8

Asp Arg Val Tyr Ile His

1 5

<210> 9

<211> 5

<212> PRT

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<223> Description of Artificial Sequence:AII (1-5)

<400> 9

Asp Arg Val Tyr Ile

1 5

<210> 10

<211> 4

<212> PRT

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<220>

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<400> 10

Asp Arg Val Tyr

1

<210> 11

<211> 3

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence:AII (1-3)

<400> 11

Asp Arg Val

1

<210> 12

<211> 7

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue

<220>

<221> MOD_RES

<222> (2)

<223> Nle

<400> 12

Arg Xaa Tyr Ile His Pro Phe

1 5

<210> 13

<211> 7

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue

<220>

<221> MOD_RES

<222> (4)

<223> Nle

<400> 13

Arg Val Tyr Xaa His Pro Phe

1 5

<210> 14

<211> 3

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII (6-8)

<400> 14

His Pro Phe

1

<210> 15

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII (4-8)

<400> 15

Tyr Ile His Pro Phe

1 5

<210> 16

<211> 7

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue class

<220>

<221> UNSURE

<222> (1)

<223> Xaa at poistion 1 can be Arg, Lys, Ala, Orn, Ser, MeGly, D-Arg, or D-Lys

<220>

<221> UNSURE

<222> (2)

<223> Xaa at position 2 can be Val, Ala, Leu, Nle, Ile, Gly, Pro, Aib, Acp, or Tyr

<220>

<221> UNSURE

<222> (4)

<223> Xaa at position 4 can be Ile, Ala, Leu, Nle, Val, or Gly

<400> 16

Xaa Xaa Tyr Xaa His Pro Phe

1 5

<210> 17

<211> 7

<212> PRT

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<223> Description of Artificial Sequence:AII analogue

<400> 17

Arg Val Tyr Gly His Pro Phe

1 5

<210> 18

<211> 7

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue

<400> 18

Arg Val Tyr Ala His Pro Phe

1 5

<210> 19

<211> 8

<212> PRT

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<220>

<223> Description of Artificial Sequence:AII analogue 1

<400> 19

Asp Arg Val Tyr Val His Pro Phe

1 5

<210> 20

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue 2

<400> 20

Asn Arg Val Tyr Val His Pro Phe

1 5

<210> 21

<211> 11

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue 3

<400> 21

Ala Pro Gly Asp Arg Ile Tyr Val His Pro Phe

1 5 10

<210> 22

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue 4

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Glu Arg Val Tyr Ile His Pro Phe
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<210> 23

<211> 8

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<213> Artificial Sequence

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<223> Description of Artificial Sequence:AII analogue 5

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Asp Lys Val Tyr Ile His Pro Phe
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<210> 24

<211> 8

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence:AII analogue 6

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Asp Arg Ala Tyr Ile His Pro Phe

1 5

<210> 25

<211> 8

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence:AII analogue 7

<400> 25

Asp Arg Val Thr Ile His Pro Phe

1 5

<210> 26

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue 8

<400> 26

Asp Arg Val Tyr Leu His Pro Phe

1 5

<210> 27

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue 9

<400> 27

Asp Arg Val Tyr Ile Arg Pro Phe

1 5

<210> 28

<211> 8

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence:AII analogue 10

<400> 28

Asp Arg Val Tyr Ile His Ala Phe

1 5

<210> 29

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue 11

<400> 29

Asp Arg Val Tyr Ile His Pro Tyr

1 5

<210> 30

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue 12

<400> 30

Pro Arg Val Tyr Ile His Pro Phe

1 5

<210> 31

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue 13

<400> 31

Asp Arg Pro Tyr Ile His Pro Phe

1 5

RUL

<210> 32

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue 14

<220>

<221> MOD_RES

<222> (4)

<223> PHOSPHORYLATION

<400> 32

Asp Arg Val Tyr Ile His Pro Phe

1 5

<210> 33

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue 15

<220>

<221> MOD_RES

<222> (3)

<223> Nle

<400> 33

Asp Arg Xaa Tyr Ile His Pro Phe

1 5

<210> 34

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<213> Artificial Sequence

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<223> Description of Artificial Sequence:AII analogue 16

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<221> MOD_RES

<222> (5)

<223> Nle

<400> 34

Asp Arg Val Tyr Xaa His Pro Phe
1 5

<210> 35

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue 17

<220>

<221> MOD_RES

<222> (4)

<223> homo Ser

<400> 35

Asp Arg Val Ser Tyr Ile His Pro Phe
1 5

<210> 36

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:p-aminophenylalanine 6 AII

<220>

<221> MOD_RES

<222> (6)

<223> p-aminophenylalanine

<400> 36

Asp Arg Val Tyr Ile Xaa Pro Phe
1 5

<210> 37

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:angiotensin I

<400> 37

Asp Arg Val Tyr Ile His Pro Phe His Leu

1 5 10

INTERNATIONAL SEARCH REPORT

Inter inal Application No
PCT/US 99/03772

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/08 C07K7/14 C12N5/06 C12N5/08 C12N5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	L. LAFLAMME ET AL.: "Angiotensin II induction of neurite outgrowth by AT2 receptors in NG108-15 cells" JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS), vol. 271, no. 37, September 1996, pages 22729-22735, XP002106039 MD US see page 22733, column 2, paragraph 1 ---	1-21
A,P	WO 98 32457 A (DIZEREGA GERE ;RODGERS KATHLEEN E (US); UNIV SOUTHERN CALIFORNIA () 30 July 1998 ---	
A	US 5 015 629 A (DIZEREGA GERE S) 14 May 1991 -----	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 June 1999	28/06/1999

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 1,5,9

The scope of claims 1,5 and 9 is unduly broad and speculative. A formula consisting virtually of variables cannot be considered to be a clear and concise definition of patentable subject-matter. (Art. 6 PCT).
Furthermore, the available experimental data only comprise a very small part of the compounds claimed, therefore the claims are also not adequately supported by the description.
Therefore, a meaningful and economically feasible search could not encompass the complete subject-matter ofthe claims. Consequently the search has been limited to Angiotensin I, II and (closely) related analogues, that is those encompassed by claims 13 and 14. (Art. 17(2)(a)(ii) PCT).